Novel Histochemical Approaches to the Prealbumin-Related Senile and Familial Forms of Systemic Amyloidosis

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The immunoperoxidase method, the autoclave method, and a newly developed alkaline-guanidine method were used to distinguish senile (SSA) and familial types (FAP) of prealbumin-related amyloidosis in formalin-fixed, paraffin-embedded tissue sections. Because all the amyloid deposits of SSA and FAP reacted positively with the anti-prealbumin antiserum, a classification of the amyloid fibril proteins of FAP and SSA by immunohistochemistry, using polyclonal anti-prealbumin antisera, was not feasible. Both the senile and familial forms of amyloidosis showed unchanged Congophilia after prolonged autoclaving. In the alkaline-guanidine method, FAP amyloids were resistant to incubation for 2 hours. On the other hand, amyloid deposits of SSA lost the Congophilia and green birefringence with 2 hours' alkaline-guanidine treatment. Therefore, the autoclave method combined with the alkaline-guanidine method will considerably facilitate differentiation of SSA and FAP, without specific antisera. (Am J Pathol 1986, 123:407–412)

THERE IS considerable diversity in chemical and immunologic properties of systemic amyloidosis, and there are at least three clinically and chemically different groups of systemic amyloidosis[1,2]: 1) systemic amyloidosis in immunocytic dyscrasia (primary and myeloma-associated amyloidosis), in which the amyloid fibril protein is the AL type; 2) reactive (secondary) systemic amyloidosis, in which the fibril protein is the AA type; and 3) familial amyloid polyneuropathy (FAP)—all of which appear to contain prealbumin or related variants.[3–5] In addition to these three types, Pitkänen et al[6] proposed senile systemic amyloidosis (SSA) as a fourth distinct type. In SSA, amyloid fibril protein (protein ASc1), which is closely related to prealbumin, is the main subunit of the fibrils.[7,8] Immunohistochemically, amyloid deposits of SSA react positively with anti-prealbumin antiserum such as FAP. Such being the case, a differentiation of SSA from FAP using only immunohistochemical techniques cannot be made.

We have now succeeded in differentiating prealbumin-related senile and familial forms of systemic amyloidosis by applying two histochemical techniques to paraffin-embedded tissue sections; one is the autoclave method,[9] and the other is a newly developed alkaline-guanidine method.

Materials and Methods

Selection of SSA

In cardiac tissues from 112 autopsies of individuals over 80 years of age, we found 12 amyloid-laden areas located in the left ventricles. One of the 12 cases, however, had AA type amyloid deposits. Eleven amyloid-laden patients were selected because paraffin blocks of several different tissues were available and there was no familial onset of amyloidosis in these subjects. The mean age of the 11 was 90.1 years (range, 80–102 years). SSA was recognized by immunostaining with the anti-prealbumin antiserum.

Selection of Familial Systemic Amyloidosis

This group included 3 subjects with FAP and one with familial amyloidosis and vitreous opacities.[10,11]

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These subjects with FAP had lived at Arao City in Japan and showed typical clinical features of Portugese type amyloid polyneuropathy.[12] The one case of familial amyloidosis with vitreous opacities was reported to be a different type of familial primary amyloidosis in Japan.

All amyloid-containing tissues were fixed in 10% formalin for over 1 week, embedded in paraffin, and cut into 5-μ sections.

Immunohistochemical Method

Human prealbumin was purified by a modification of the method of Rask et al.[13] Briefly, prealbumin was obtained from human plasma by DEAE-Sephadex and Sephadex G 200 chromatography at low ion strength. Antiserum against human prealbumin was raised in rabbits.[9] Specificity of this antiserum was checked by Western blotting, which was performed as follows. Normal human serum was treated overnight with 6 M guanidine-HCl containing 5% mercaptoethanol and then dialysed thoroughly. Guanidine-treated serum and nontreated human serum were diluted by addition of sodium dodecyl sulfate (SDS) electrophoresis sample buffer with 5% mercaptoethanol and heated at 100 C for 3 minutes. The low-molecular-weight calibration kit from Pharmacia Fine Chemicals was used for standard proteins. Electrophoresis was performed in 15% polyacrylamide gels, according to Laemmli.[14] Proteins were electrophoretically transferred to nitrocellulose sheets. Before detection with affinity reagents, nonspecific binding sites on the blots were blocked by incubation for 2 hours with 10% normal goat serum. The blotted proteins were incubated for 1 hour at room temperature with a 1:1000 dilution of anti-prealbumin antiserum. The following procedures were performed with avidin–biotin system reagents (Vector Laboratories, Burlingame, Calif).

For immunohistochemistry, the unlabeled antibody avidin–biotin complex (ABC) method, in which peroxidase was used as the enzyme, was performed with the use of anti-prealbumin antiserum diluted to 1:2000 or 1:4000, as previously described.[9] Sections treated in the same manner with preimmune rabbit-serum were incubated as controls. The specificity of the reaction was established by absorbing the anti-prealbumin antiserum with purified human prealbumin.

Autoclave Method

We developed this method to classify the amyloid fibril proteins.[9] After deparaffinization, the sections were placed in Petri dishes and incubated for 30 or 120 minutes in an autoclave (Tomy, SD-30N) at 130 C (1.7 kg/sq cm). After the pressure in the autoclave had decreased to an atmospheric level and the temperature had fallen below 60 C, the sections were removed and stained with alkaline Congo red.[15] A duplicate slide section was stained with alkaline Congo red alone for direct comparison.

Alkaline-Guanidine Method

After deparaffinization, the sections were incubated for 1 minute or 2 hours with 7 M guanidine-HCl (Nakarai Chemicals, Kyoto, a specially prepared reagent for biochemical use) dissolved in 0.1 N NaOH. The temperature was below 4 C, and the pH 12.6 and 12.8. Before dissolving guanidine-HCl, it was dried by vacuum pumping for the correct weight. After the treatment, the tissue sections were thoroughly washed with tap water, stained with alkaline Congo red, and examined by polarizing microscopy.

Results

Immunohistochemical Analyses

Results of Western blotting are shown in Figure 1. The anti-prealbumin antiserum reacted on a single band

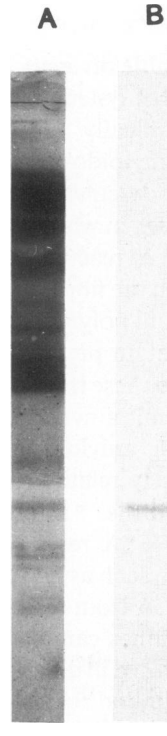


Figure 1—Western blotting of guanidine-treated normal human serum. **A**—Normal human serum proteins stained with Amido black. **B**—Immunoblot using anti-prealbumin antiserum. A single reactive band was apparent.

Table 1—Data on Immunostaining, Autoclaving, and Alkaline-Guanidine Methods for Classifying Prealbumin-Related Senile and Familial Amyloidosis

Case	Age (years)	Sex	Examined organ	Immunostain (anti-PreA)	Autoclave			Guanidine		
					Pre	30 min	120 min	Pre	1 min	120 min
SSA										
1	85	F	Heart	+	+ + +	+ + +	+ + +	+ + +	+ +	–
2	82	M	Heart	+	+ + +	+ + +	+ + +	+ + +	+ + +	–
3	83	M	Heart	+	+ + +	+ + +	+ + +	+ + +	+ +	–
			Esophagus	+	+ + +	+ + +	+ +	+ + +	+ +	–
4	80	M	Heart	+	+ + +	+ + +	+ + +	+ + +	+ + +	–
			Lung	+	+ + +	+ + +	+ +	+ + +	+ +	–
5	102	M	Heart	+	+ + +	+ + +	+ +	+ + +	+ +	–
6	93	F	Heart	+	+ + +	+ + +	+ + +	+ + +	+ + +	–
			Lung	+	+ + +	+ + +	+ + +	+ + +	+ +	–
7	93	F	Heart	+	+ + +	+ + +	+ + +	+ + +	+ + +	–
			Tongue	+	+ + +	+ + +	+ +	+ + +	+ +	–
8	95	F	Heart	+	+ + +	+ + +	+ + +	+ + +	+ + +	–
9	90	M	Heart	+	+ + +	+ + +	+ +	+ + +	+ +	–
			Tongue	+	+ + +	+ + +	+ +	+ + +	+ +	–
10	91	F	Heart	+	+ + +	+ + +	+ +	+ + +	+ +	–
			Lung	+	+ + +	+ + +	+ + +	+ + +	+ +	–
11	97	F	Heart	+	+ + +	+ + +	+ + +	+ + +	+ +	–
			Intestine	+	+ + +	+ + +	+ + +	+ + +	+ +	–
FAP										
12	35	F	Nerve	+	+ + +	+ + +	+ +	+ + +	+ + +	+ +
13	45	M	Nerve	+	+ + +	+ + +	+ + +	+ + +	+ + +	+ +
14	53	M	Kidney	+	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
15	41	M	Heart	+	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +

Anti-PreA, anti-prealbumin. Immunostaining: +, positive. Autoclave and guanidine method: + + +, unchanged pattern of green birefringence compared with pretreatment; + +, slight decrease of green birefringence; +, marked decrease of green birefringence; –, loss of green birefringence.

(Figure 1B), about 14 kd, among the blotted proteins of guanidine-treated serum proteins (Figure 1A). In nontreated serum proteins, two reactive bands, about 14 and 54 kd, were detected (data not shown).

Table 1 shows the results of immunostaining on tissue sections from 15 subjects with systemic amyloidosis. The amyloid deposits of SSA and familial amyloidosis showed a strong reactivity with the anti-prealbumin antiserum when the ABC method was used. This positive staining occurred in the same distribution as amyloid stained with Congo red and showed a green birefringence under polarized light (Figures 2 and 3). Absorption of anti-prealbumin antiserum with purified human prealbumin blocked the positive staining of amyloid. The preimmune rabbit serum, at the same dilution, showed negative staining of amyloid deposits.

Autoclave Method

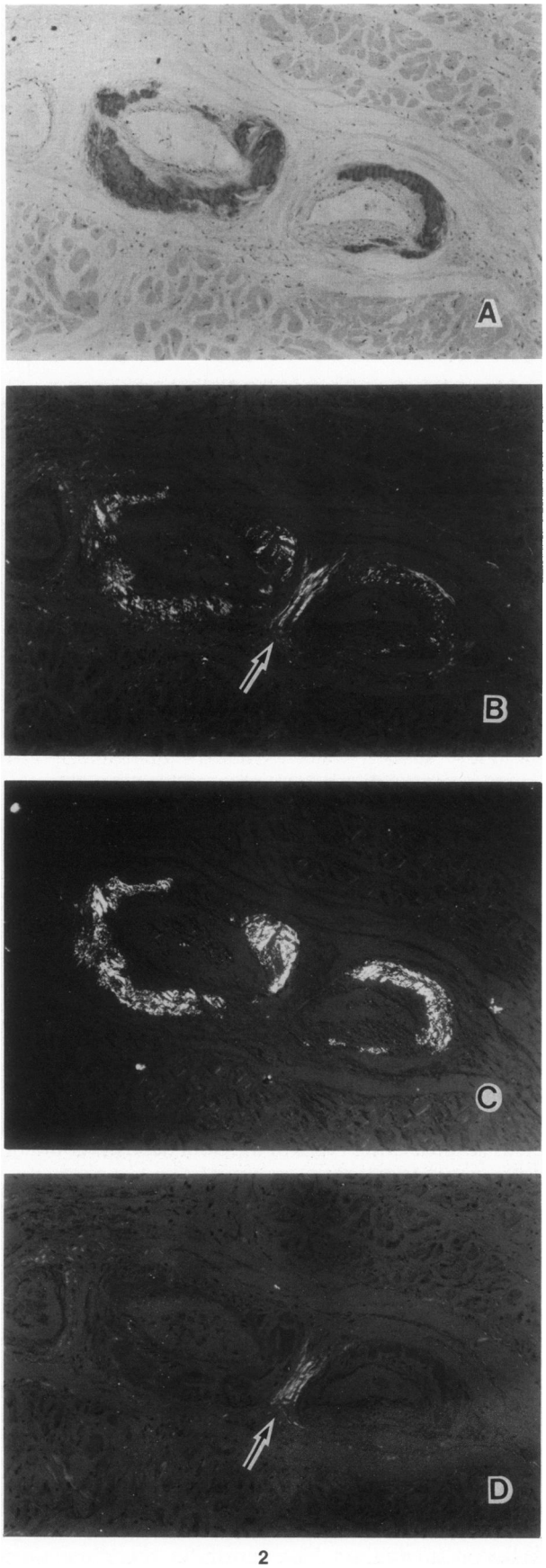
The results obtained by the autoclave method are summarized in Table 1. After 2 hours' autoclaving at 130 C, the amyloid deposits of SSA and familial amyloidosis were stained positively with Congo red and showed a green birefringence under polarized light (Figures 2 and 3). Compared with pretreatment findings, the autoclaved sections had an unchanged green birefringence.

Alkaline-Guanidine Method

Table 1 shows the results of alkaline-guanidine treatment. After alkaline-guanidine treatment for 1 minute, both senile and familial forms of systemic amyloidosis were stained positively with Congo red. When the duration of the treatment was prolonged to 2 hours, the amyloid deposits of SSA lost the Congophilia and the green birefringence (Figure 2). However, Congophilia in familial amyloidosis did not change or decreased only slightly, compared with pretreatment (Figure 3). FAP amyloids had a preserved Congophilia even after 4 and 10 hours' treatment; however, we chose 2 hours' treatment because other types of amyloid deposits could be differentiated clearly.

Discussion

Senile systemic amyloidosis, often known as "senile cardiac amyloidosis," is proposed as a fourth distinct type of systemic amyloidosis.[6] The amyloid fibril protein of SSA is mainly composed of protein AScl, which is closely related to human prealbumin.[7,8,16] Prealbumin is also the main fibril protein in many forms of FAP.[3-5] Therefore, we tried to differentiate prealbumin-related senile and familial forms of systemic amyloi-



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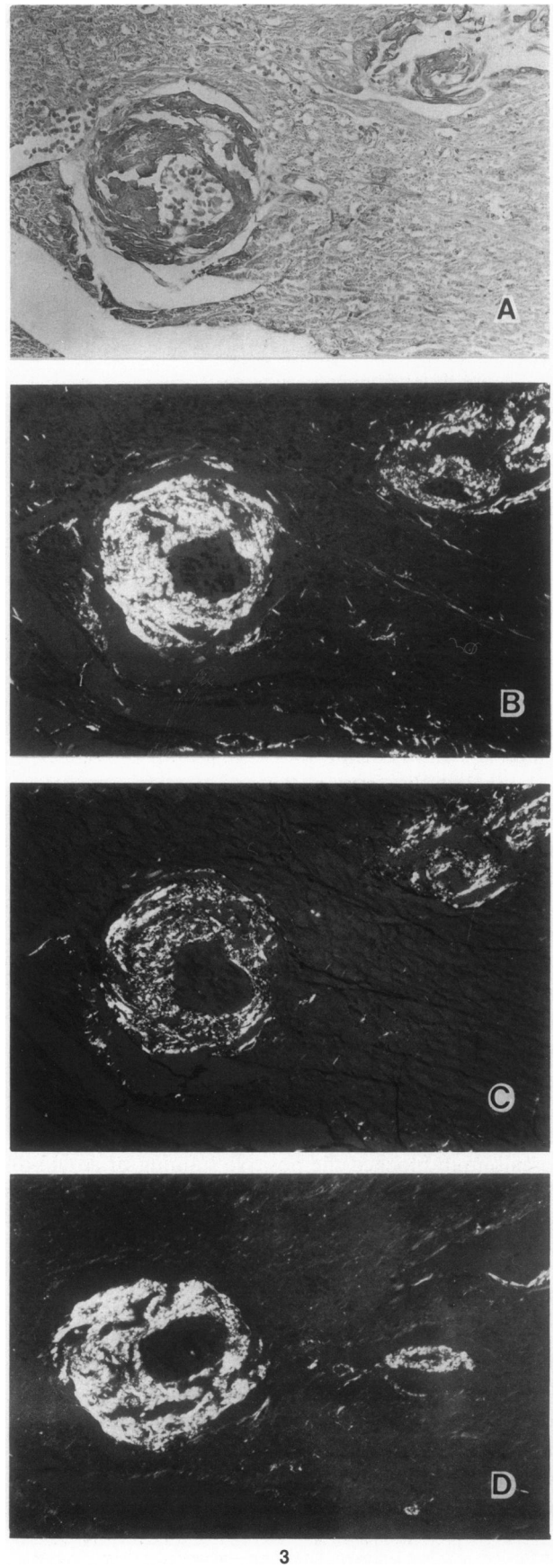

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Table 2—Comparison of Autoclaving, Alkaline–Guanidine, and Immunostaining Methods for Assessing Systemic Amyloidosis

Amyloid typing	Autoclaving at 130 C			Alkaline–guanidine			Immunostain (anti-PreA)
	Pre	30 min	120 min	Pre	1 min	120 min	
AA	+ + +	−	−	+ + +	−	−	−
AL	+ + +	+ +	±	+ + +	+ +	±	−
FAP	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+
SSA	+ + +	+ + +	+ + +	+ + +	+ +	−	+

Anti-PreA, anti-prealbumin antiserum. Autoclaving and alkaline–guanidine methods; + + +, unchanged pattern of green birefringence compared with pretreatment; + +, slight decrease of green birefringence; ±, marked decrease or loss of green birefringence; −, loss of green birefringence. Immunostaining: +, positive, −, negative.

dosis by the use of immunohistochemical methods and our histochemical techniques.

Regarding the specificity of the immunohistochemical method, the anti-prealbumin antiserum shared two bands of nontreated serum proteins and a single band of guanidine-treated serum proteins. Fex et al[17] reported that human prealbumin separated into two bands, monomer and tetramer, in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) containing mercaptoethanol and made a single band, a monomer, in SDS-PAGE after dissociation by dialysis against guanidine-HCl. Thus, our results for Western blotting showed that the anti-prealbumin antiserum reacted specifically with human prealbumin. In the immunostaining, the anti-prealbumin antiserum reacted positively with amyloid deposits of both senile and familial forms. Our results show that the immunohistochemical method using monospecific anti-prealbumin cannot separate senile and familial forms of systemic amyloidosis. Cornwell et al[8] reported that the anti-prealbumin reaction was completely blocked by purified protein ASc1, and the reaction of anti-ASc1 was similarly blocked by purified human prealbumin. So, at present, a classification of the amyloid fibril proteins of senile and familial types by immunohistochemistry using polyclonal anti-prealbumin or anti-ASc1 antibodies is not feasible.

Data on the histochemical methods are shown in Table 2. In the autoclave method,[9] AA type amyloidosis lost the affinity of Congo red after 30 minutes' autoclaving. AL type and FAP can be differentiated by 2 hours' treatment. However, we could not classify prealbumin-related amyloidosis because amyloid deposits of both SSA and FAP were resistant to 2 hours' autoclaving.

In a newly developed alkaline-guanidine method, three major types of systemic amyloidosis were clearly differentiated (T. Tashima, T. Kitamoto, J. Tateishi, manuscript in preparation). After 2 hours' alkaline-guanidine treatment, amyloid deposits of SSA lost Congophilia-like AL type amyloidosis, while Congophilia in amyloid deposits of FAP was unchanged or only slightly decreased. Delellis et al[18] reported that Congophilia of amyloid deposits in tissue sections was lost by 6 M guanidine-HCl in 0.1 M PBS, pH 7.2, for 45 minutes at room temperature. We used this procedure, and few sections lost Congophilia. The tissues used by Delellis et al were unfixed or fixed in 10% formalin for 24–48 hours, whereas the tissues we used had been fixed for over 1 week. Thus, the different responses may be due to the prolonged fixation. In purification protocols of amyloid fibril proteins, buffered guanidine or alkaline-guanidine treatments have generally been used for dissolving amyloid fibrils.[19,20] Therefore, amyloid deposits in tissue sections may be denaturated by alkaline-guanidine, and amyloid fibrils may lose the binding sites for Congo red. This reaction occurred in a short time in the case of AA protein and a longer time in the cases of AL protein and protein ASc1. We consider two possible explanations as to the different reactions to alkaline-guanidine treatment between the senile and familial forms of prealbumin-related amyloidosis. First, the different reactions may be due to a concentration effect, ie, more prealbumin present in FAP amyloids. However, Felding et al[16] reported that the prealbumin-like protein (13 kd) was a major component in both SSA and FAP amyloid fibrils. The second explanation is that the conformation of FAP amyloid fibrils may be different from that in SSA. FAP amyloids in the Japanese are composed of a prealbumin variant which contains a methionine for valine substitution at Position 30.[21] This substitution may change the tertiary structure of prealbumin and the conformation of FAP amyloid fibrils composed of polymerized abnormal prealbumin. Protein ASc1 has a discrepant character which is resistant to physical denaturation (autoclaving), but sensitive to chemical denaturation (alkaline-guanidine). The reason for this discrepancy

Figure 2—Senile form of systemic amyloidisis. **A**—Anti-prealbumin antiserum reacted positively with amyloid deposits in cardiac vessels. Counterstained with hematoxylin. **B**—Adjacent section was stained with Congo red. Peroxidase-positive materials in **A** showed green birefringence. *Arrow* shows collagen. **C**—After 2 hours' autoclaving, amyloid deposits showed unchanged green birefringence. **D**—After 2 hours' alkaline–guanidine treatment, peroxidase positive amyloid deposits lost the Congophilia. *Arrow* shows collagen. (×75)

Figure 3—Familial form of systemic amyloidosis. **A**—Anti-prealbumin antiserum reacted positively with amyloid deposits in cardiac vessels. Counterstained with hematoxylin. **B**—Cardiac tissue section was stained with Congo red. Peroxidase-positive materials showed green birefringence. **C**—After 2 hours' autoclaving, amyloid deposits showed unchanged green birefringence. **D**—After 2 hours' alkaline-guanidine treatment, amyloid deposits showed an unchanged pattern. (×150)

remains obscure; however, SSA can be differentiated from FAP by making use of this characteristic of protein ASc1.

The application of the autoclave method combined with the alkaline-guanidine method should be a useful tool when one is attempting to differentiate types of amyloid fibril proteins. Our methods will facilitate simple and rapid differentiation of amyloids in biopsy materials without specific antisera, as well as retrospective studies on preserved tissues embedded in paraffin.

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